1-1-1999

Characterization and autoproteolytic activity of N[superscript pro] of bovine viral diarrhea virus

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Characterization and autoproteolytic activity of N^{pro} of bovine viral diarrhea virus

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Microbiology

Major Professor: Kenneth B. Platt

Iowa State University

Ames, Iowa

1999

Graduate College

Iowa State University

This is to certify that the Master's thesis of

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ABSTRACT

The nonstructural N-terminal protease (N^{pro}) of the pestiviruses possesses autocatalytic activity that cleaves itself from the remainder of the viral polyprotein. The function of N^{pro} in virus replication and the catalytic unit responsible for self-cleavage have not been determined. N^{pro} nucleotide sequence was obtained from Genbank or by sequence analysis for 5 isolates of each of the following pestiviruses: classical swine fever virus, border disease virus, bovine viral diarrhea virus type 1, and bovine viral diarrhea virus type 2. The N^{pro} sequences of all 20 isolates were compared and conserved amino acid residues were identified. A conserved Gly-Ser-Asp-Gly motif was identified in N^{pro} that resembles the Gly-Asp-Ser-Gly active site motif of serine proteases. Site-directed mutagenesis of conserved regions of the bovine viral diarrhea virus strain 890 N^{pro} was accomplished by PCR using mutagenic primers. Additional random mutations were generated by error prone PCR amplification. Mutated N^{pro} sequences were cloned into pBluescript for T7 RNA polymerase directed transcription. Autoprotease activity of mutated N^{pro} proteins was evaluated based on the presence or absence of a 20 kDa cleaved protein (N^{pro}) from the 37 kDa precursor after *in vitro* translation. Mutations at amino acids Gly67, Cys69, Ser124, and Tyr129 resulted in loss of N^{pro} cleavage. Rümenapf *et al.* (1998) had previously

reported that His49 was essential for N^{pro} cleavage. In this study, mutations at Gly67 and Cys69 may have disrupted the catalytic function of Asp68. In addition, the conserved motif surrounding Ser124 and prevention of cleavage of N^{pro} by mutation of Ser124 suggest that N^{pro} is a serine protease resembling chymotrypsin with a His49/Asp68/Ser124 catalytic triad.

GENERAL INTRODUCTION

Introduction

Bovine viral diarrhea virus (BVDV) is a member of the Pestivirus genus of the *Flaviviridae* family. This genus includes three viruses that infect livestock populations worldwide. Members of the genus include classical swine fever virus (CSFV) or hog cholera virus (HCV) of swine, border disease virus (BDV) of sheep, and BVDV of cattle. BVDV is an important pathogen of cattle that has been implicated in many disease processes including reproductive failure, respiratory disease, and enteric disease.

Pestiviruses are enveloped viral particles about 50 nm in diameter. The pestivirus genome consists of positive sense, single stranded RNA that is 12.3 kb in length for noncytopathic isolates. Cytopathic isolates have a genome that is about 12.5 kb in length or longer. At the 5' and 3' ends of the viral genome are untranslated regions, 360 to 385 nucleotides and 186 to 229 nucleotides in length, respectively. The pestivirus genome undergoes cap-independent translation via an internal ribosome entry site in the 5' untranslated region. The genome encodes one open reading frame that is translated into a polyprotein of nearly 4000 amino acid residues. This polyprotein is processed co- and posttranslationally by viral-encoded and host

cell proteases resulting in 11 to 12 mature proteins: N^{pro}, C, E^{rns}, E1, E2, p7, NS2/3 (or NS2 and NS3), NS4A, NS4B, NS5A, and NS5B.

BVDV has been divided into two biotypes, noncytopathic and cytopathic, based upon its effect on cultured cells. In addition, BVDV can be separated into genotypes BVDV1 and BVDV2. BVDV1 has been further divided into subgenotypes BVDV1a and BVDV1b. Sequence variations in the 5' untranslated region are used to group viral isolates into genotypes and subgenotypes.

The first translated product of the pestivirus genome is N^{pro}, which has autoproteolytic activity. This protein cleaves itself from the remainder of the viral polypeptide, although the purpose of its release and its role in replication are unknown. The N^{pro} occurs only in pestiviruses and has no counterpart in other members of the *Flaviviridae* family. Previous studies have determined the cleavage site of N^{pro} of CSFV by sequencing the N-terminal end of the adjacent downstream encoded C protein. A conserved region immediately upstream of the cleavage site was found among all pestiviruses, indicating that auto-catalytic cleavage by N^{pro} occurred at the same site for all pestiviruses. Comparisons of cellular and viral-encoded proteases with N^{pro} identified some sequence homology between papain-related cysteine proteases and N^{pro}. In previous experiments, some conserved amino acids

were mutated to identify catalytic residues associated with N^{pro}, but the catalytic unit was not identified.

Thesis Organization

This thesis is organized in the standard format consisting of a literature review that is divided into five sections (clinical aspects of BVDV, molecular biology of BVDV, previous analysis of N^{pro}, viral proteases, and rationale and research aims), materials and methods, results, discussion, general conclusions, and references cited.

REVIEW OF THE LITERATURE

Clinical aspects of BVDV

History

The appearance of a highly contagious and transmissible disease of cattle was first reported in 1946 in New York and named virus diarrhea (Olafson *et al.,* 1946). This disease was clinically characterized by fever, leukopenia, nasal discharge, depression, anorexia, dehydration, abortion, and diarrhea. Gross lesions observed in diseased cattle included ulcers and necrosis of the mucous membranes of the lips, cheeks, tongue, pharynx, esophagus, abomasum, and cecum. The disease was later found to be caused by a virus (Prichard *et al.,* 1955; Schipper *et al.,* 1955). Since its discovery, bovine viral diarrhea virus (BVDV) has been implicated in many disease processes including reproductive failure, fetal defects, respiratory disease, and enteric disease (Baker, 1995). BVDV is an important cause of economic losses for cattle producers worldwide.

Pestivirus genus

BVDV is a member of the *Pestivirus* genus of the *Flaviviridae* family. The *Flaviviridae* family includes flaviviruses (viruses that induce dengue fever,

yellow fever, and Japanese encephalitis), pestiviruses, and hepatitis C virus. The pestivirus genus contains three viral groups that infect livestock populations worldwide. Members of this genus include classical swine fever virus (CSFV) or hog cholera virus (HCV) of swine, border disease virus (BDV) of sheep, and bovine viral diarrhea virus (BVDV) of cattle (Wengler *et al.,* 1995).

Pestivirus transmission

BVDV is transmitted between animals by inhalation or ingestion of infected saliva, oculonasal discharge, urine, or feces (Dufell and Harkness, 1985). In addition, infection through semen of acutely or persistently infected bulls has been shown (Stober, 1984). Contaminated hypodermic needles may also serve as viral transmitters (Roeder and Harkness, 1986).

Pestiviruses were originally named after the species from which they were first isolated, but cross-infection between species has been observed. BVDV can infect cattle, sheep (Carlsson, 1991), and swine (Carbrey *et al.,* 1976; Terpstra and Wensvoort, 1988). BDV and BVDV have been reported to infect both sheep and cattle (Dufell and Harkness, 1985). Cattle persistently infected (discussed below) with BVDV were reported to cause outbreaks of disease in sheep when placed in close contact with the sheep (Carlsson, 1991). BVDV may produce clinical signs and lesions in swine

similar to those observed in chronic CSFV infections in swine (Terpstra and Wensvoort, 1988). Swine are the only species shown to be naturally infected with CSFV, indicating that CSFV is species specific (Loan and Storm, 1968; Terpstra, 1991).

Subacute infection

It has been estimated that 70% to 90 % of BVDV infections in adult cattle are subclinical (Ames, 1986). These cattle may have a mild elevation in body temperature, leukopenia, and decrease in milk production (Moerman *et al.,* 1994). These subclinical infections induce antibody production that confers protection from clinical disease for life. Antibody may be detected as early as 2 to 4 weeks after infection. However, these antibodies do not protect the fetus from infection. The absence of clinical signs in a pregnant dam does not indicate fetal protection, as BVDV has been shown to cross the placenta and infect the fetus with nearly 100% efficiency (Duffell and Harkness, 1985). Consequences of fetal infection are described below.

Acute infection

Disease caused by BVDV infection is usually mild and relatively brief. The virus has been shown to replicate primarily in the upper respiratory tract and lymphoid tissue (Bolin, 1990). An incubation period of 5 to 7 days

precedes the onset of clinical signs (Duffell and Harkness, 1985). Viremia is usually detected 4 to 5 days post-inoculation and may persist for up to 15 days (Brownlie *et al.,* 1987; Duffell and Harkness, 1985). Clinical signs include depression, fever, anorexia, nasal and ocular discharge, and diarrhea. Decreased lymphocyte numbers (Bolin *et al.,* 1985b; Markham and Ramnaraine, 1985; Roth *et al.,* 1986), and decreased neutrophil (Roth *et al.,* 1981) and monocyte (Ketelsen *et al.,* 1979) function also has been reported. Oral erosions have been observed on occasion. BVDV infection has been shown to stimulate production of high titres of neutralizing antibodies (Donis *et al.,* 1988; Bolin *et al.,* 1991a) which peak 8-10 weeks after inoculation (Nettleton and Entrican, 1995). These antibodies can persist for years and provide protection from disease and reinfection (Baker, 1995; Howard *et al.,* 1989, 1994; Potgieter, 1995). Immunosuppression has been reported during BVDV infection. The speculated result of this immunosuppression is an exacerbation of a secondary infection caused by parainfluenza virus type 3 (PI-3), infectious bovine rhinotracheitis (IBR) virus, coronavirus, rotavirus, *Pasteurella* spp., *Salmonella* spp., or coccidia (Baker, 1987).

Persistent infection

BVDV is divided into two biotypes based on its effect on cell culture: noncytopathic and cytopathic (Lee and Gillespie, 1957; Gillespie *et al.,* 1960). Noncytopathic BVDV accounts for more than 90% of BVDV infections in cattle, and is considered to be the "standard" biotype for BVDV (Dubovi, 1992). Infection of pregnant cattle by BVDV may induce fetal death (Cassaro *et al.,* 1971; Done *et al.,* 1980; Duffell and Harkness, 1985; Kendrick, 1971), abortions (Ames, 1986; Gillespie *et al.,* 1967; Stober, 1984; Ward *et al.,* 1969), stillbirths (Woodard, 1994), congenital defects (Brown *et al.,* 1973, 1974, 1975; Cassaro *et al.,* 1971; Done *et al.,* 1980; Duffell and Harkness, 1985; Wilson *et al.,* 1984), the birth of small, underweight calves, or the birth of persistently infected (PI) young (McClurkin *et al.,* 1984; Straver *et al.,* 1983), depending on the viral strain and the stage of gestation when infection occurs. Infection of the fetus with a cytopathic BVDV may result in stillbirth or birth of a calf with antibody against BVDV (Brownlie *et al.*, 1989; Duffell and Harkness, 1985). Infection of a fetus during the first 120 days of gestation with a noncytopathic BVDV strain can result in the birth of a persistently infected animal (McClurkin *et al.*, 1984). PI animals are immunotolerant to the infecting strain, continually shed virus, and do not produce neutralizing antibodies to the infecting strain (Houe and Meyling, 1991; McClurkin *et al.,* 1984; Moerman *et al.*, 1993; Taylor *et al.*, 1997).

Some PI calves may be born weak and have slow growth rates, while other PI calves appear healthy (Barber *et al.,* 1985; Coria and McClurkin, 1978; Cutlip *et al.,* 1980).

Mucosal disease

Mucosal disease is a lethal manifestation induced by BVDV virus, resulting from superinfection of a PI calf with a cytopathic BVDV (Bolin *et al.*, 1985a; Brownlie *et al.*, 1984; Moennig *et al.,* 1990). Although similar clinical signs exist in both viral diarrhea and mucosal disease, there are distinct differences. Viral diarrhea is a disease characterized by mild to moderate clinical signs with high morbidity and low mortality rates. However, mucosal disease is characterized by severe clinical signs of enteric disease with low morbidity and high mortality rates. Initial studies reported extensive lesions of the oral cavity, Peyer's patches, colon, and mucosa that ultimately lead to death (Ramsey and Chivers, 1953).

Hemorrhagic disease

Hemorrhagic disease is reported to be another consequence of BVDV infection. Infection by virulent noncytopathic BVDV causes severe disease and has been implicated in outbreaks in the United States (Corapi *et al.,* 1989; Rebhun *et al.,* 1989), Canada (Carman *et al.,* 1998; Pellerin *et al.,*

1994) and Great Britain (David *et al.,* 1994). Clinical signs include fever, pneumonia, diarrhea, and sudden death in cattle of all ages (Carman *et al.*, 1998). Multiple ulcerations of the oral cavity, intestinal mucosa, upper alimentary tract, esophagus and rumen were reported. Experimental reproduction of this disease in calves has been performed and the clinical signs observed included fever, diarrhea, depression, leukopenia, lymphopenia, neutropenia, and thrombocytopenia (Bolin and Ridpath, 1992; Ellis *et al.,* 1998).

Molecular biology of BVDV

Virion and genome

The Pestiviruses are enveloped viral particles about 50 nm in diameter. The genome consists of positive sense, single stranded RNA 12.3 kb in length for noncytopathic isolates (Deng and Brock, 1992; Ishikawa *et al.,* 1995; Meyers *et al.,* 1989a; Moormann *et al.,* 1996) and 12.5 kb in length or longer for cytopathic isolates (Collett *et al.,* 1988a; DeMoerlooze *et al.,* 1993; Renard *et al.,* 1985; Roath and Berry, 1997). The difference in lengths is attributed to inserts in the nonstructural protein NS3 of the cytopathic viruses (discussed below). At the 5' and the 3' ends of the genome are untranslated

regions (UTR) of 360 to 385 nucleotides and 186 to 229 nucleotides in length, respectively (Collett, 1992).

BVDV genotypes

Two genotypes of BVDV, BVDV1 and BVDV2, are recognized based on sequences of the 5' UTR (Pellerin *et al.,* 1994; Ridpath *et al.,* 1994). In addition, BVDV1 has been further divided into subgenotypes BVDV1a and BVDV1b (Pellerin *et al.,* 1994; Ridpath and Bolin, 1998). Although the 5' UTR is the most conserved region among pestiviruses, slight sequence variations in the 5' UTR were used to group viral isolates into genotypes and subgenotypes. Previous studies have shown that antigenic differences exist between BVDV1 and BVDV2 isolates (Ridpath *et al.,* 1994). These antigenic variations may explain why vaccination against viruses from one genotype does not fully protect from infection by viruses of the other genotype. BVDV1 viruses include classical BVDV isolates used for vaccine production, diagnostic tests, and research as well as numerous uncharacterized field isolates. BVDV2 viruses are primarily recent isolates from fetal bovine sera, persistently infected calves born to dams vaccinated against BVDV, and cattle that had died from hemorrhagic syndrome (Bolin and Ridpath, 1998; Pellerin *et al.,* 1994; Ridpath *et al.,* 1994). Additionally, segregation of pestivirus isolates into genotypes has been accomplished by sequence

analysis of the N-terminal protease (Becher *et al.,* 1997) and envelope protein 2 (van Rijn *et al.,* 1997). These genotype segregations are similar to ones based on the 5' UTR.

Translation initiation

Although the 5' UTR contains six AUG codons, translation does not start until nucleotide 386 (Collett *et al.,* 1988a). Cell-free translation studies failed to demonstrate products from these small open reading frames (Wiskerchen *et al.,* 1991). The genome is believed to be translated in a cap-independent manner (Brock *et al.,* 1992; Poole *et al.,* 1995; Wiskerchen *et al.,* 1991; Rijnbrand *et al.,* 1997). In addition, analysis of the 5' UTR of pestiviruses has revealed complex secondary structures comparable to those found in picornaviruses. Multiple AUG codons and secondary structure predictions indicate that the 5' UTR of Pestiviruses contains an internal ribosome entry site (IRES) on the 5' end of the UTR for translation initiation (Rice, 1996; Rijnbrand *et al.,* 1997; Poole *et al.,* 1995). In addition, a pseudoknot located immediately upstream of the translation initiation codon is also important for ribosome binding (Le *et al.,* 1995). The combination of the IRES and the pseudoknot directs translational initiation to the authentic initiation codon (Rijnbrand *et al.,* 1997).

Open reading frame

The viral RNA encodes one large open reading frame, which is translated into a polyprotein of nearly 4000 amino acid residues. This polyprotein is processed co- and posttranslationally by both viral-encoded and host cell proteases resulting in 11 to 12 mature proteins (Stark *et al.,* 1990; Wiskerchen and Collett, 1991; Wiskerchen *et al.,* 1991). Figure 1 depicts the genomic organization of BVDV. In addition to the unique 5' UTR characteristics described above, the 3' UTR is not polyadenylated (Meyers and Thiel, 1996).

N^{pro}

The first translated product is the non-structural protein N^{pro}, that has been shown to have autoproteolytic activity (Stark *et al.,* 1993; Wiskerchen *et al.,* 1991). This protein cleaves itself from the remainder of the viral polypeptide, although the purpose of its release and the role in virus replication are unknown. The N^{pro} has no counterpart in other Flaviviruses. Studies have determined the cleavage site of the N^{pro} of CSFV by sequencing the N-terminal end of the capsid protein (Stark *et al.,* 1993). A conserved region among all pestiviruses is located immediately upstream of the cleavage site, indicating that autocatalytic cleavage by N^{pro} occurs at the same site for all pestiviruses. Amino acid comparisons of N^{pro} with cellular

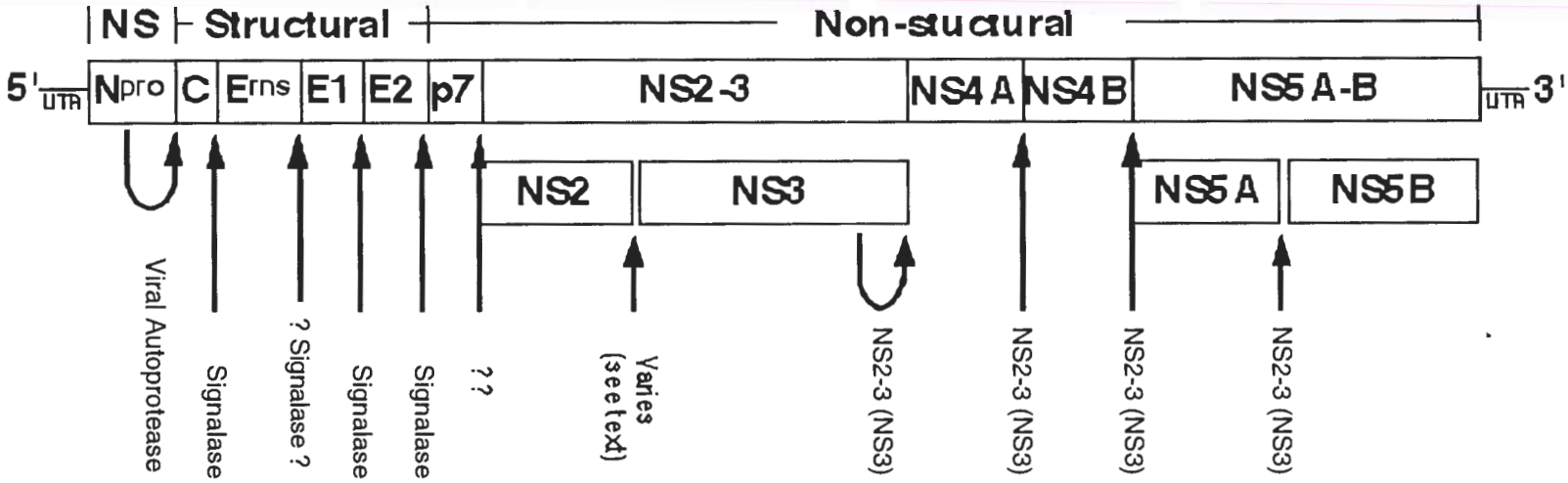

Figure 1. Genomic organization of pestiviruses. The 5' and 3' untranslated regions (UTR) and viral proteins encoded by the open reading frame are shown. The protease involved in processing each protein is listed and arrows indicate the site of cleavage. "?" indicates that the protease responsible for cleavage is not known.

and viral-encoded proteases have suggested that N^{pro} has some similarity to papain-related cysteine proteases (Gorbalenya *et al.,* 1991; Stark *et al.,* 1993; Wiskerchen *et al.,* 1991).

Structural proteins

After translation of N^{pro}, the capsid protein (C) is translated, followed by three envelope glycoproteins: E^{rns}, E1, E2. Immediately after the C protein, an internal signal sequence mediates translocation of the envelope proteins to the lumen of the endoplasmic reticulum. The cleavage of the C protein from the proceeding envelope precursor protein E^{rns}/E1/E2 occurs upon translocation of the envelope precursor protein into the endoplasmic reticulum and is mediated by a host cell signalase (Rümenapf *et al.,* 1993). In addition, the E^{rns}/E1/E2 precursor is cleaved into E^{rns}/E1 and E2 by a host cell signalase. The cleavage of E^{rns}/E1 into E^{rns} and E1 is delayed. Cleavage site analysis of E1 revealed that a protease other than a host cell signalase may be required and this is likely to be the reason for the cleavage delay (Rümenapf *et al.,* 1993). The type of protease that cleaves E^{rns}/E1 into E^{rns} and E1 remains unknown. It has been hypothesized that E1 may be transported into the Golgi apparatus for processing by this unknown protease (Rümenapf *et al.,* 1993).

Envelope proteins E1 and E2 have membrane anchors that allow integration into the lipid envelope of the virion (Rümenapf *et al.,* 1993; van Zijl *et al.,* 1991), but the method of association of E^{rns} with the lipid envelope is unknown. All three glycoproteins form disulfide-linked complexes: E^{rns} homodimer, E1-E2 heterodimer, and E2 homodimer (Thiel *et al.,* 1991; Weiland *et al.,* 1990). These complexes are assembled because cysteine residues in the C- terminal half of the E2 form intermolecular disulfide bonds with E1 or E2. Similarly, cysteine residues in the N-terminal half of E2 form intramolecular disulfide bonds (van Rijn *et al.,* 1994).

Studies have demonstrated neutralizing epitope activity of the E^{rns} and E2 (Onisk *et al.,* 1991; Weiland *et al.,* 1990; Weiland *et al.,* 1992; Xue *et al.,* 1990). In addition, E^{rns} and E2 of CSFV induce a protective immunity in the host against challenge-exposure with virulent classical swine fever virus (Hulst *et al.,* 1993; König *et al.,* 1995; van Zijl *et al.,* 1991). The E1 glycoprotein lies buried beneath the envelope and does not induce antibody production (Weiland *et al.,* 1990).

Envelope glycoprotein E^{rns} from CSFV has been shown to have RNase activity (Schneider *et al.,* 1993). Proteins with similar RNase activity have not been found in Flaviviruses other than the pestiviruses. A search of known genetic sequences revealed significant sequence similarity between E^{rns} and certain fungal and plant ribonucleases (Hulst *et al.,* 1994; Schneider

et al., 1993). Biochemical characterization of E^{rns} revealed substrate specificity for uridine and zinc was found to inhibit the RNase activity of E^{rns} (Schneider *et al.,* 1993). Later studies showed that the antibodies, which effectively blocked the infection of cells *in vitro*, also exerted E^{rns} RNase inhibition (Windisch *et al.,* 1996). These results suggested that E^{rns} may have a vital role in the life cycle of the virus, possibly participating in RNA processing, although the specific function of this RNase activity with respect to the virus life cycle is not yet understood.

Envelope glycoprotein E2 has been shown to induce a protective immune response in studies with CSFV (Hulst *et al.,* 1993; König *et al.,* 1995; van Zijl *et al.,* 1991). Later studies showed that the N-terminal portion of E2 is the most immunogenic portion of the glycoprotein (van Rijn *et al.,* 1993). Although E2 is the most variable pestivirus gene, similarities between BVDV and CSFV E2 proteins were reported in cysteine residue locations and variable amino acid positions (Paton *et al.,* 1992; van Rijn *et al.,* 1994). Monoclonall antibody reactivity studies have failed to detect an E2 epitope that is conserved among all pestivirus strains (van Rijn *et al.,* 1997).

Downstream of the glycoprotein E2 is the putative p7 protein. This 7kDa protein, although not a major structural constituent, links glycoprotein E2 to nonstructural protein NS2-3 in the polyprotein. Viral protein expression studies identified that the E2-p7 cleavage is often incomplete, giving rise to a

larger E2-p7 product. E2-p7 cleavage is believed to occur via host cell signalase (Elbers *et al.,* 1996).

Non-structural proteins

The remainder of the Pestivirus open reading frame encodes nonstructural (NS) proteins. Translation of the precursor protein NS2-3 and its role in cytopathogenicity of the virus are discussed later. Cleavage events are mediated at four sites (NS3-4A, NS4A-4B, NS4B-5A, NS5A-5B) by a viral-encoded serine protease (Tautz *et al.,* 1997; Wiskerchen and Collett, 1991; Xu *et al.,* 1997). The N-terminal end of NS3 codes for this serine protease, which releases itself at the C-terminal end of NS3. The NS3 is responsible for the release of NS4A, NS4B, NS5A, and NS5B (Wiskerchen and Collett, 1991). It was later reported that NS3 requires NS4A as a cofactor for cleavage at two sites: NS4B-NS5A and NS5A-NS5B (Xu *et al.,* 1997). In addition to serine protease activity, the NS3 also exhibits NTPase and helicase activity (Tamura *et al.,* 1993; Warrener and Collett, 1995; Wiskerchen and Collett, 1991).

NS3 and cytopathogenicity

Experiments have shown that noncytopathic pestiviruses produce only the NS2-3 precursor protein (Collett *et al.,* 1988b). Cytopathic effects of

pestiviruses can be attributed to cleavage of the NS2-3 precursor protein to yield the NS3 protein (Donis and Dubovi, 1987a,b; Greiser-Wilke *et al.,* 1992; Purchio *et al.,* 1984). NS3 production and subsequent cytopathogenicity have been linked to the following mutational events: insertion of cellular RNA sequences, duplication and rearrangement of pestivirus sequences, and large in-frame deletions resulting in subgenomic defective interfering (DI) RNAs (Meyers and Thiel, 1996). It has been hypothesized that recombinational events cause the integration of cellular and viral coding sequences into the viral genome. The newly inserted sequences that result in a cytopathic virus either exhibit autoprotease activity, serve as a recognition site for protease cleavage, or induce a conformational change in which NS2-3 cleavage occurs by some unknown mechanism. Cytopathic BVDV strains with cellular inserts probably gained the cellular sequence through template switching during the synthesis of viral negative strand RNA. This recombination may be guided by nucleotide interactions between the newly synthesized viral RNA and cellular or viral RNA molecules (Meyers and Thiel, 1996).

BVDV isolate NADL was reported to contain a 270 nucleotide cellular insertion of bovine mRNA into the NS2-3 region (Meyers *et al.*, 1990). A later study (Mendez *et al.,* 1998) showed that deletion of this cellular insert (cIns) from the NADL isolate genome produced viable virus particles, but cytopathogenicity had been abolished and NS3 protein was not detectable.

In addition, deletion of the cIns resulted in lower levels of viral RNA synthesis, although virus protein and virion production was not affected. The role of cIns in cleavage of NS2-3 is not known (Mendez *et al.,* 1998).

Another intriguing association is the cellular insert of light chain 3 of microtubule-associated proteins 1A and 1B into the NS2-3 of cytopathic BVDV strain JaCP (Meyers *et al.,* 1998). An insertion of light chain 3 sequence, flanked by viral duplicated elements was able to induce cleavage of NS3. Both insertion of light chain 3 and viral duplications were necessary to produce viable virus particles (Meyers *et al.,* 1998). In a separate study, cytopathic BVDV strain CP7 was found to contain a 27 nucleotide viral duplication in its NS2-3. Experimental deletion of this duplicated viral sequence resulted in the loss of NS2-3 cleavage (Tautz *et al.,* 1996).

BVDV isolates have been reported to contain ubiquitin inserts. A 228 nucleotide insertion in BVDV Osloss was found to encode a complete copy of ubiquitin (Meyers *et al.*, 1989b). A study of BVDV isolate CP1 revealed a 366 nucleotide insertion composed of a complete ubiquitin gene plus a truncated portion of a ubiquitin gene embedded in a 2384 nucleotide viral duplication (Meyers *et al.,* 1991). The in-frame insertion of ubiquitin upstream of the NS3 protein provides a processing site for cellular ubiquitin carboxyl-terminal hydrolase to release the NS3 protein.

Cytopathic BVDV strains Pe515CP and CP6 were found to contain viral inserts as well as viral sequence duplications. The viral insert for both isolates was found to be N^{pro}. For isolate Pe515CP, the N^{pro} was missing the first 14 N-terminal amino acids, while isolate CP6 contained a complete N^{pro} coding sequence. Both N^{pro} inserts were flanked by large duplicated viral sequences derived from the NS2-3 encoding region. The large duplicated regions of Pe515CP and CP6 were found to be 2304 and 3635 nucleotides, respectively. The N^{pro} of both of these isolates was functional, resulting in autoproteolytic removal of itself from the NS2-3 region and generating the amino terminus of the NS3. Despite the absence of the first 14 amino acids of the Pe515CP N^{pro} insert, the protease retained its autoproteolytic activity (Meyers *et al.,* 1992).

The frequent association of the N-terminus of the NS3 protein as a target for nonhomologous recombination has lead to speculation that this site may be highly prone to recombination (Meyers and Thiel, 1996). A recent study found the contrary. Viral RNA was extracted from BVDV infected cells and sequenced. Results demonstrated that recombination events had occurred at many different genomic locations (Desport *et al.,* 1998). Thus, clustering of recombinational events at the N-terminal end of NS3 was not supported by this analysis. Apparently, nonhomologous recombination in pestiviruses is determined more by RNA secondary structure than by

sequence. As RNA polymerase moves along the template, it may be slowed by processivity of the enzyme or template secondary structure features, and disassociate from the template. The RNA polymerase may associate with a new template and continue RNA synthesis. The release of the RNA polymerase from the new template and the return to the original viral template also may be influenced by template secondary structure or polymerase pausing. Template switching events are likely to produce nonviable genomes because of frameshifts and the introduction of nonsense codons. The relatively high frequency of recombinational events may cause rapid evolution of the Pestivirus genome (Desport *et al.,* 1998).

An increased genome size caused by duplications and/or insertions is associated with cytopathic BVDV viruses. Truncated BVDV genomes have also been described (Kupfermann *et al.,* 1996; Tautz *et al.,* 1994). Truncated genomes are associated with defective interfering particles (DI). These DIs cannot replicate without the aid of a helper virus that compensates for missing portions of the DI truncated genome. BVDV isolate CP9 contains an 8 kb genome, as the result of a deletion that extends from the C-terminal end of the N^{pro} through the NS2. The genome of this DI does not encode any structural proteins, but is packaged in the structural proteins produced by a noncytopathic helper BVDV. The autoproteolytic activity of N^{pro} contained in the DI was shown to generate the N-terminus of the adjacent NS3, resulting

in cytopathogenicity (Tautz *et al.,* 1994). A second DI particle, CP13, has been reported (Kupfermann *et al.,* 1996). This DI contains two internal deletions that removed the structural genes N^{pro}, p7, and NS2. Replication of CP13 is dependent on aid from a noncytopathic helper virus.

Cytopathic BVDV isolates Oregon C24V and Singer were reported to not possess genomic alterations and were thought to generate NS3 by processing of the NS2-3 precursor. It was determined that the C-terminal portion of the NS2 and the first 66 amino acids of NS3 play a vital role in NS2-3 cleavage. Analysis of the NS2 C-terminal ends of strains Oregon and Singer with respect to NS2 of other cytopathic isolates revealed single amino acid differences that may be responsible for cleavage of NS2-3 to generate NS3 without genome alteration. Nucleotide point mutations have resulted in these amino acid changes that may serve as recognition sites for protease cleavage (Kummerer *et al.,* 1998).

NS4 and NS5

The role of NS4A and NS4B in viral replication is not known. However, for hepatitis C virus, a member of the flavivirus family, the analogous NS4A serves as a cofactor for enzymatic activity of NS3 (Rice, 1996). NS5B contains a characteristic amino acid sequence motif Gly-Asp-Asp, which indicates that it encodes for an RNA-dependent RNA polymerase (Meyers *et*

al., 1989a). Studies have shown that the NS5 protein possesses RNA-dependent RNA polymerase activity (Tan *et al.,* 1996). Recent studies have shown that NS5 of BVDV, yellow fever virus, and hepatitis C virus (all members of the flavivirus family) is phosphorylated by host cell serine/threonine kinases (Reed *et al.,* 1998). Phosphorylation may regulate the subcellular localization of NS5 to the nucleus for altered host gene expression by the virally encoded RNA polymerase. Phosphorylated NS5 may also enhance viral replication through interactions with NS3 or other viral or host proteins. Phosphorylation of NS5 has also been proposed to modulate the host interferon antiviral response (Reed *et al.,* 1998).

Previous analysis of N^{pro}

Because a combination of viral and host proteases are necessary to process the BVDV precursor into mature proteins, it is important to investigate the viral proteases to determine their role in the production of virions. Discussed below are studies that have attempted to determine the function of N^{pro}, a self-cleaving protease of unknown importance in replication of pestiviruses.

The autoproteolytic activity of the N^{pro} of pestiviruses has been demonstrated (Wiskerchen *et al.,* 1991; Stark *et al.,* 1993; Thiel *et al.,* 1993).

In the earliest study, characterization studies were performed using amino acid mutagenesis (Wiskerchen *et al.,* 1991). Here, two BVDV genome constructs were made and expressed, one encoding the N^{pro} and a truncated C, and a second encoding the N^{pro}, C, E^{rns}, and E1 regions. The N^{pro} was shown to cleave itself from both expressed polypeptides, indicating that downstream translation is not required. In addition, these results suggest that translation of downstream viral genes does not signal for the release of the N^{pro}.

A review of common features of viral proteases did not reveal any amino acid sequence similarity between the N^{pro} of BVDV and other viral proteases. An earlier study showed that histidine, aspartic acid, serine, and cysteine were enzymatically functional amino acids commonly found in viral proteases (Wellinnk and van Kammen, 1988). In addition, glycine, serine, alanine, threonine, methionine, and valine were common N-terminal amino acids created by proteolytic cleavage. Citing this work, Wiskerchen *et al.* (1991) made site-directed mutations in the N^{pro} encoding region to determine enzymatically active amino acids. Three mutations resulted in the loss of a N^{pro} product: His40, His49, and Trp164. Mutations at amino acid residues Ser124, Ser169, Thr171, Glu173, Glu175, and Lys179 did not affect p20 cleavage. Deletions were also made, Δ111-124 and Δ163-173, eliminating cleavage of the N^{pro}. Based on these findings, they concluded that because

the Trp164 mutation resulted in loss of N^{pro} function, N^{pro} might resemble chymotrypsin (Wellink and van Kammen, 1988) and the alphavirus capsid autoproteinase (Strauss and Strauss, 1986; Strauss *et al.,* 1987), both serine proteases that cleave at a Trp residue. Additionally, because the amino acid after Trp164 in BVDV is a Val residue, and Val was reported to be frequently created as an new N terminal end by viral proteases (Wellink and van Kammen, 1988), Val165 was predicted to be the newly created N-terminal amino acid of the C protein. Thus, the preliminary characterization of the N^{pro} of BVDV revealed similarity to the cleavage pattern of the capsid autoproteinase of alphaviruses and suggested that cleavage occurred after Trp164 (Wiskerchen *et al.,* 1991). However, the definitive protease class of N^{pro} or the cleavage site between N^{pro} and C were not established.

Additional studies have been performed on the 23 kDa N^{pro} of CSFV, which is analogous to the 20 kDa N^{pro} of BVDV (Stark *et al.,* 1993). The N^{pro} is the first protein encoded by the CSFV open reading frame, followed by the capsid protein (C). In previous studies, CSFV genome constructs of N^{pro} (23 kDa) and C (14 kDa) failed to demonstrate a 37 kDa precursor protein, leading to speculation that the N^{pro} contained autoproteolytic activity (Thiel *et al.*, 1991). Cleavage between the N^{pro} and C regions was reported later, with N^{pro} possessing the autoproteolytic activity (Stark *et al.,* 1993).

Studies were performed to determine the cleavage site of the N^{pro} of CSFV (Stark *et al.,* 1993). The amino acid sequence surrounding the putative cleavage site of N^{pro} contains only two Cys residues: Cys161 and Cys168. Depending on the actual cleavage site of N^{pro}, Cys radiolabeling would reveal where cleavage occurs. If N^{pro} cleavage occurred at Trp164, as had been previously assumed (Wiskerchen *et al.,* 1991), both N^{pro} and C would be radiolabeled in this experiment. However, if N^{pro} cleavage occurred after Cys168, only N^{pro} would be radiolabeled. *In vitro* translation was performed in the presence of [^{35}S]Cys. Only a single translational product 23 kDa in size was radioactively labeled, indicating that cleavage did not occur between Trp164 and Val165, as previously assumed. It was concluded that the cleavage site of N^{pro} was located downstream from Trp164. Stop codons were introduced at various positions, but only stop codons downstream of Cys168 resulted in a 23 kDa protein that co-migrated with the native CSFV N^{pro} protein. N-terminal amino acid sequencing of the capsid protein was also performed, revealing a serine on the N-terminal end. Based on these results, the cleavage event was determined to occur between the N^{pro} and C proteins between residues Cys168 - Ser169.

A recent study was conducted to identify essential amino acids for N^{pro} activity (Rümenapf *et al.*, 1998). Protease inhibitors were used to classify the type of protease to which N^{pro} belonged. Serine, cysteine, and aspartic acid

protease inhibitors were not successful in preventing N^{pro} cleavage, indicating that N^{pro} does not belong to any of these protease types. Site directed mutagenesis revealed that Glu22, His49, and Cys69 were essential for proteolytic activity. This data confirmed a previous hypothesis that His49 was an enzymatically important residue (Wiskerchen *et al.,* 1991). His130, which previously had been hypothesized to be an enzymatically important amino acid (Stark *et al.,* 1993), did not prevent N^{pro} cleavage when mutated. Mutational analysis of histidine residues His40 and His99 did not prevent N^{pro} cleavage. Cysteine residues were also mutated without affecting N^{pro} cleavage: Cys112, Cys134, Cys138, and Cys161. These results suggested that N^{pro} was not a papain-like cysteine protease. Additionally, mutation of Asp68 did not prevent N^{pro} cleavage. N-terminal deletions of N^{pro} revealed that the first 21 amino acids were not essential to proteolytic function. Results also suggested that Glu22 was a catalytic amino acid residue, indicating that the association of Glu22-His49-Cys69 might represent a catalytic triad similar to the subtilisin-like serine proteases.

Because the function of N^{pro} is unknown, studies have been performed to determine its role in viral replication. In one study, N^{pro} was not required for viral replication in cell culture (Tratschin *et al.,* 1998). Replacement of the CSFV N^{pro} with ubiquitin resulted in recombinant virus particles that were able to infect cell culture as well as induce anti-CSFV antibodies when inoculated

into pigs. Deletion of the entire N^{pro}, but leaving an AUGGGG starting sequence, did not result in infectious virus. These results suggested that the N^{pro} has no essential role in viral replication other than autocatalytic cleavage at its carboxy-terminus to generate the amino-terminus of the viral capsid protein (Tratschin *et al.,* 1998).

In another study, the BVDV DI9c genome was found to contain the 5' UTR, N^{pro}, NS3 to NS5B and the 3' UTR (Behrens *et al.,* 1998). Experimental deletion of the N^{pro} destroyed the ability of DI9c particles to replicate. Replacement of the N^{pro} with ubiquitin in-frame with the remainder of the DI genome also rendered the genome nonreplicative. When the N-terminal 42 amino acids (126 nucleotides) of the N^{pro} were fused to a ubiquitin gene, the adjacent NS3 was cleaved and DI RNA was able to replicate. These results suggested that an essential structural and functional part of the IRES transcription element was located within the N^{pro}. In addition, these results contradicted previous data that had suggested that N^{pro} was not required for viral replication (Tratschin *et al.,* 1998). Similar IRES transcription *cis*-acting elements had been previously described from the flaviviruses Kunjin (Khromykh and Westaway, 1997) and hepatitis C virus (Lu and Wimmer, 1996). The 5' end of N^{pro} (first 126 nucleotides) may prove to be a *cis*-acting element that is critical to viral RNA replication in BVDV (Behrens *et al.,* 1998).

Viral proteases

Serine proteases

Serine proteases are characterized by a serine (Ser) residue in the active site. Two additional active amino acids, aspartic acid (Asp) and histidine (His), are highly conserved in the three largest serine protease clans to form a catalytic triad capable of cleaving peptide bonds (Rawlings and Barrett, 1994). Arrangement of amino acids in the catalytic triad separates the serine proteases into clans. Despite the different arrangements of amino acid residues in the catalytic triad, glycine (Gly) residues tend to be conserved around the catalytic serine in a Gly-X-Ser-Gly motif. Chymotrypsin-like serine proteases are secreted proteins that contain a His/Asp/Ser catalytic triad (Rawlings and Barrett, 1994). Subtilisin-like serine proteases preferentially cleave at aromatic residues [phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr)] but will cleave a nonaromatic or charged side chain if a hydrophobic residue is present three or four positions upstream of the cleavage site. The subtilisin catalytic triad order is Asp/His/Ser (Rawlings and Barrett, 1994). Table 1 lists the serine protease clans and the amino acids that form the catalytic triads.

The capsid shell of herpesviruses, such as cytomegalovirus (CMV), is assembled by proteolytic processing of scaffolding proteins within the capsid

core. The herpesviruses encode a protease (PR) at the N terminal end of their large genome that processes these scaffolding proteins. This virally encoded protease is highly conserved among all herpesviruses, although there is little sequence homology to known serine proteases. Cytomegalovirus protease crystal structure (Qiu *et al.,* 1996) revealed a novel fold that contains an active site His/His/Ser. Structural similarities between the cytomegalovirus protease and chymotrypsin, a serine protease, indicate that the herpesvirus protease is a serine protease.

Table 1. Serine proteases are divided into two major clans: chymotrypsin and subtilisin. Viral proteases that have been characterized are listed with their catalytic triad sequence.

	Protease type	**Active residues**
Chymotrypsin Clan	Serine	His/Asp/Ser
Alphavirus, Sindbis virus capsid	Serine	His/Asp/Ser
Flavivirus NS3	Serine	His/Asp/Ser
Potyvirus P1	Serine	His/Asp/Ser
Arterivirus	Serine	His/Asp/Ser
Herpesvirus, Cytomegalovirus PR	Serine	His/His/Ser
Subtilisin Clan	Serine	Asp/His/Ser
Herpes virus 1	Serine	Asp/His/Ser

Sindbis virus, an alphavirus, codes for a core protein (SCP) that cleaves itself from the viral polyprotein. After this autocatalytic event, the core protein inactivates itself by inserting its C-terminal tryptophan into the substrate-binding site. The core protein then assembles into an icosahedral core that surrounds the viral RNA. A His/Asp/Ser catalytic triad, similar to chymotrypsin, was discovered with an active serine residue (Tong *et al.*, 1993).

The flavivirus non-structural protein NS3 cleaves non-structural proteins at the C terminal end of the viral polypeptide. NS3 lacks sequence homology with any known protease, but analysis of crystallized NS3 revealed a three-dimensional structure similar to chymotrypsin, with a His/Asp/Ser catalytic triad (Kim *et al.* 1996). Other members of the chymotrypsin-like serine protease clan include the N-terminal protease P1 of the potyviruses and the Nsp4 replicase processing peptidase of arteriviruses (Rawlings and Barrett, 1994).

Members of the subtilisin clan of serine proteases can be grouped by the order of their catalytic triad: Asp/His/Ser. They also differ substantially in their three-dimensional structure when compared to the chymotrypsin clan. The only known viral member of the subtilisin-like proteases is a catfish herpes virus protease (Rawlings and Barrett, 1994).

Cysteine proteases

Cysteine proteases that resemble the cellular enzyme papain have been reported for many positive stranded RNA viruses. A list of viral cysteine proteases and their associated catalytic units is provided in Table 2. Essential cysteine and histidine residues have been identified in the potyvirus CI protein (Oh and Carrington, 1989), alphavirus nsP2 (Hardy and Strauss, 1989) and coronavirus p28 (Baker *et al.*, 1993). Two groups of cysteine proteases were proposed (Gorbalenya *et al.*, 1991). The first group contains

Table 2. Cysteine proteases that have been identified from viruses are listed. The amino acid residues involved in proteolytic activity and their sequential order varies.

	Protease type	**Active residues**
Papain	Cysteine	Asn/His/Cys
Alphavirus nsP2	Cysteine	Cys/His
Adenovirus	Cysteine	His/Glu/Cys
Aphthovirus L-pro	Cysteine	His/Cys
Coronavirus p28	Cysteine	Cys/His
Picornavirus 2A	Cysteine	His/Asp/Cys
Picornavirus 3C	Cysteine	His/Glu/Cys (His/Cys dyad?)
Potyvirus HC-Pro	Cysteine	Cys/His
Potyvirus NIa	Cysteine	His/Asp/Cys
Rhinovirus 2A	Cysteine	His/Asp/Cys

accessory leader proteases that autocatalytically cleave at their own C terminal end. Potyviruses, rhinoviruses, and aphthoviruses are members of this accessory leader protease group (Dougherty and Semler, 1993; Sommergruber *et al.,* 1997). The second group of viral cysteine proteases process nonstructural proteins that aid in viral replication. These members include the proteases of alphaviruses, rubiviruses, and coronaviruses (Dougherty and Semler, 1993).

Adenoviruses code for a cysteine protease that processes the core proteins within an assembled virion. The active site of this protease contains a His/Glu/Cys catalytic triad. An 11 amino acid peptide cofactor increases protease activity 300-fold by altering the active site conformation (Ding *et al.,* 1996). In addition, contact with viral DNA increases enzyme activity an additional 20-fold.

An N-terminal autoprotease, also called leader proteinase (L-pro), is found in aphthoviruses, of the *Picornaviridae* family. This protease was found to be a papain-related protease by mutational analysis, with Cys-23 and His-120 being catalytic residues (Piccone *et al.,* 1995a). The leader protease was not required for viral replication (Piccone *et al.,* 1995b), but was a participant in cleavage of host cell initiation factor 4G and termination of host cell cap-dependent protein synthesis (Devany *et al.,* 1988).

The 3C protease of picornaviruses is responsible for the cleavage of nonstructural proteins. The protease also has been shown to bind viral RNA and to aid in initiating RNA replication. Computer generated secondary structure predictions and structural analysis of crystallized 3C cysteine protease concluded that 3C resembled the serine protease chymotrypsin (Matthews *et al.,* 1994). The 3C protease shares less than 10% sequence homology with members of the chymotrypsin family of serine proteases (Bazán and Fletterick, 1988). A 3C catalytic triad of His/Glu/Cys is positioned similar to the His/Asp/Ser catalytic triad of serine proteases. The 3C proteases may also utilize a weaker His/Cys catalytic dyad that is similar to the caspase His/Cys dyad. This protease represents a distinct group of cysteine proteases that structurally resembles the serine protease chymotrypsin.

Rationale and research aims

Much remains unknown about the N^{pro} of BVDV and its autoproteolytic activity. The N^{pro} may only generate the amino terminus of the adjacent capsid protein, or it may cleave or aid in cleavage at other sites within the polyprotein. It is also possible that N^{pro} may cleave cellular proteins or interact with other opportunistic pathogens. The cleavage site of N^{pro} has

been determined (Stark *et al.,* 1993) but the purpose for autocatalytic activity and its role in viral pathogenesis are unknown. In addition, enzymatically important amino acids need to be identified in order to characterize the protease.

Previous studies of the N^{pro} of BVDV (Wiskerchen *et al.,* 1991) and CSFV (Stark *et al.,* 1993) both stated the need to classify the N- terminal proteases of pestiviruses. Additionally, both studies called for the identification of catalytic amino acids. Previously, histidine, aspartic acid, serine, and cysteine were identified as amino acids important for viral protease function (Wellink and van Kammen, 1988). Additionally, amino acid comparison of pestivirus N^{pro} to other cellular and viral proteases revealed conserved amino acids (Stark *et al.,* 1993). Similarities were reported between the pestiviral N^{pro} and papain-like cysteine proteases (Stark *et al.,* 1993).

This study compares the N^{pro} region of BVDV type 1 and 2, CSFV, and BDV to other cellular and viral proteases in order to identify conserved regions and enzymatically active areas within N^{pro}. Based on these conserved regions, mutational analysis of conserved amino acids should indicate enzymatically functional amino acids. In addition, this study attempts to characterize the N^{pro} of BVDV by identifying its catalytic unit.

If the protease resembles a cysteine protease, amino acids Gly67, Cys69, His130 and Cys168 likely represent enzymatically functional residues of the N^{pro}. Mutational substitution of these residues should destroy the autoproteolytic activity of the N^{pro} autoprotease. Additional mutations made within conserved regions of N^{pro} might reveal which amino acids are important for cleavage and establish a possible protease characterization. The purpose of this study is to determine the effect that mutations at amino acids Gly67, Cys69, His130 and Cys168 have on protease function of N^{pro} and the effects of additional mutations at other sites in N^{pro} have on cleavage of N^{pro} from C.

MATERIALS AND METHODS

Cells and viruses

BVDV type 1 isolates included: C24V (Gillespie *et al.,* 1960), NY-1, Singer, TGAN (Bolin and Ridpath, 1992), and Van Meter (Ridpath and Bolin, 1990). BVDV type 2 isolates included: 1373, 28508, 296nc, AZSPLN, Parker, and 890 (Bolin and Ridpath, 1992). BVDV type 1 and 2 isolates were propagated in bovine turbinate (BT) cells grown in McCoy's medium (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum that had been tested and found to be free of BVDV and BVDV antibodies (Bolin *et al.,* 1991b). BT cells were grown in 25 cm^2 flasks until 80% confluent. The media was removed from the flask and virus was added to a concentration of 10^5-10^6 cell culture infectious dose virus as previously described (Ridpath and Bolin, 1991). After one hour incubation, the inoculum was replaced with McCoy's media and the inoculated cells were incubated at 37°C for 24 (cytopathic isolates) or 48 hours (noncytopathic isolates) before being frozen at -80°C for storage. BDV isolates were grown in ovine fetal turbinate (OFTU) cells using the same media and supplements for 48 hours. BDV isolates included: CB2, CB5, Idaho207, Idaho209, and Idaho211 (Ridpath, personal communication). BVDV and BDV isolates used for this study included laboratory reference strains, vaccine strains, and field strains

isolated from diseased cattle and sheep. All isolates had been maintained and stored at the National Animal Disease Center.

RNA extraction

Viral RNA was extracted from infected cells that were freeze/thawed using Catrimox-14 (Iowa Biotechnology Corp., Oakdale, IA, USA) as previously described (Dahle and Macfarlane, 1993). Flasks of frozen (-80°C) virus infected cells were thawed at room temperature and the resulting freeze/thaw lysate was used for viral RNA extraction. One ml of Catrimox-14 (36 mg/ml H_2O), 50 μg of yeast tRNA (Life Technologies, Gaithersburg, MD, USA), and 400 μl of freeze/thaw cell lysate were mixed, vortexed and incubated for 10 minutes at room temperature. The solution was centrifuged at 15,000 x **g** for 10 minutes and the supernatant was discarded. The pellet was resuspended in 500μl of 2M LiCl and then centrifuged at 15,000 x **g** for 10 minutes. The resulting pellet was washed twice with cold 75% EtOH and centrifuged at 15,000 x **g** for ten minutes after each wash. After the final wash and spin, the RNA pellet was dried and resuspended in 50 μl H_2O. The RNA was either used immediately for RT-PCR or stored at -80°C.

RT-PCR

For comparison of the N^{pro} of pestivirus isolates, the region encoding the N^{pro} of each isolate was amplified by RT-PCR. Primers were selected from conserved genomic regions of BVDV1 (CP7 GenBank accession #U63479; NADL GenBank accession #M31182), BVDV2 (890 GenBank accession #U18059), and BDV (X818 GenBank accession #AF037405; BD31 GenBank accession #U70263) that flanked the N^{pro} of each isolate group using the Primer 2 program (Scientific and Educational Software, State Line, PA, USA). The primers were synthesized by IDT Inc. (Coralville, IA, USA). The region encoding the N^{pro} was selectively amplified by adding 2 µl of the viral RNA to 98 µl of reverse transcriptase polymerase chain reaction (RT/PCR) mix [1x PCR reaction buffer containing 1.75 mM $MgCl_2$, 50 mM Tris-HCl pH 9.2, 16 mM $(NH_4)_2SO_4$ (Boehringer Mannheim Corp., Indianapolis, IN, USA), 0.1 mM dNTP (Amresco, Solon, OH, USA), 100 pM of each primer (forward and reverse), 50 units MMLV reverse transcriptase (Gibco/BRL, Gaithersburg, MD, USA), 200 units RNase inhibitor (Promega, Madison, WI, USA) and 3 units of Expand High Fidelity polymerase (Boehringer Mannheim Corp., Indianapolis, IN, USA)]. The cycling conditions were as follows: reverse transcriptase reaction (56°C for 1 hour; 94°C for 1 minute), PCR reaction (94°C for 15 seconds; 50°C for 30 seconds; 72°C for 2 minutes) for 35 cycles and one cycle of 72°C for 10 minutes.

Products were visualized on 1% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, ME, USA) with 0.5 μg/ml ethidium bromide (Amresco, Solon, OH, USA) to verify proper fragment sizes.

Nucleotide sequencing analysis for N^{pro} comparison

The N^{pro} sequence of each pestivirus isolate was obtained by direct sequencing of the PCR products. PCR amplicons were partially purified through Microcon-100 microconcentrator spin columns (Amicon, Inc., Beverly, MA, USA). To each column, 100 μl of PCR product and 300 μl of H_2O were added. The columns were centrifuged at 1500 x **g** for 4 minutes. The columns were washed twice with 400 μl of H_2O and centrifuged at 1500 x **g** for 4 minutes after each wash. Purified PCR products were recovered by inverting the columns and centrifuging at 12,000 x **g** for 15 seconds. Cycle sequencing reactions were performed by adding 25 ng of purified amplicon to ThermoSequenase sequencing reaction premix (Amersham Life Science, Cleveland, OH, USA). The cycle sequencing reactions were performed by one cycle of 94°C for 3 minutes, 98°C for 30 sec; 25 cycles of 98°C for 15 seconds, 50°C for 10 seconds, 60°C for 4 minutes; one cycle of 72°C for 10 minutes. Sequencing products were purified with Centri-Sep spin-columns (Princeton Separations, Adelphia, NJ, USA). Columns were hydrated with 800 μl of H_2O and vortexed briefly to mix the matrix solution. The columns

were initially centrifuged at 850 x **g** for 2 minutes. The products from the cycle sequencing reactions were added to the column and the columns were centrifuged at 850 x **g** for 3 minutes. The purified sequencing products were desiccated by centrifugation under vacuum for 30 minutes without heat. Sequencing products were loaded and analyzed on an ABI 373 automated DNA sequencer (Applied Biosystems, Forster City, CA, USA). Polyacrylamide gels (6%) were used in the automated DNA sequencer. Gels were prepared by combining 40 g of ultra-pure urea (Amresco, Solon, OH, USA), 9.6 ml of 50% Long Ranger (FMC Bioproducts, Rockland, ME, USA), and 9.6 ml of 10x TBE. The volume of the solution was adjusted to 80 ml with H_2O and the solution was filtered through a 0.2 μ cellulose nitrate filter. The addition of 45 μl of TEMED (Amresco, Solon, OH, USA) and 400 μl of 10% ammonium persulfate (Amresco, Solon, OH, USA) initiated polymerization of the gel. The resulting sequences were edited and aligned using Mac-DNASIS (Hitachi Software Engineering Co., Ltd., San Bruno, CA, USA) and MegAlign (DNASTAR, Madison, WI, USA).

RT-PCR for cloning of N^{pro}

RT-PCR for the generation of the control N^{pro} PCR amplicon was performed using BVDV2-890 specific primers encoding a *Bam* H I restriction site at the 5' end of the insert (genome position 386) and a *Xba* I restriction

site at the 3' end of the insert (genome position 1216) by the same PCR protocol listed above. Primer sequences and their respective restriction sites are listed in Table 3. BVDV2-890 genomic RNA was used as template for the PCR reaction and amplification was performed as described above.

The resulting PCR product was purified by a Microcon-100 microconcentrator spin column (Amicon, Inc., Beverly, MA, USA) and 1 μg of the product was digested in 50 μl of reaction mix containing 10 units of *Bam* H I (Gibco BRL, Gaithersburg, MD, USA), 10 units of *Xba* I (Gibco BRL,

Table 3. Sequences of primers used to generate control N^{pro} insert and for site-directed mutagenesis PCR reactions. The restriction sites encoded by each primer are underlined.

Primer	Nucleotide sequence	Restriction site
386	GCCGCCGGATCCACCATGGAGTTGTTTTCAAATG	*Bam* H I
1216 comp	GCCGCCTCTAGACTAGTTCCACTGGGTAATATTC	*Xba* I
G67V	CCTGCCGCGGAAAGTCGACTGCCGG	*Sal* I
G67V comp	CCGGCAGTCGACTTTCCGCGGCAGG	*Sal* I
C69T	GGAAAGGTGACACGCGTAGAGGTAATG	*Mlu* I
C69T comp	CATTACCTCTACGCGTGTCACCTTTCC	*Mlu* I
H130V	GGAAATTATACGTAATCTACATCTGC	*Sna* B I
H130V comp	GCAGATGTAGATTACGTATAATTTCC	*Sna* B I
C168A	GGGTCACCAGCGCTAGCGATGAAGGGAG	*Nhe* I
C168A comp	CTCCCTTCATCGCTAGCGCTGGTGACCC	*Nhe* I
S169I	GGGTCACCAGCTGCATCGATGAAGGGAG	*Cla* I
S169I comp	CTCCCTTCATCGATGCAGCTGGTGACCC	*Cla* I

Gaithersburg, MD, USA) and 1x reaction buffer (10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) for one hour at 37°C. Plasmid pBluescript II KS (Stratagene, La Jolla, CA, USA) was also digested with *Bam* H I and *Xba* I under the same conditions. The digested products were then pooled, phenol/chloroform extracted, ethanol precipitated, and resuspended in 15 µl of H_2O to which one unit of T4 DNA ligase (Gibco BRL, Gaithersburg, MD, USA) and 4 µl of 5x T4 DNA ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% polyethylene glycol) were added for overnight ligation at 14°C. The ligated plasmid was used to transform competent DH5α *E. coli* cells (Gibco BRL, Gaithersburg, MD, USA) by mixing 10 µl of ligation reaction and 50 µl of cells and incubation at 4°C for 30 minutes. The cells were heat shocked at 42°C for 1 minute. One ml of SOC media (Sambrook *et al.,* 1989) was added and the cells and were incubated at 37°C for 30 minutes. The cells were plated (100 µl) onto Luria-Bertani (LB) agar plates supplemented with 100 µg/ml of ampicillin (Sigma Chemical, St. Louis, MO, USA) and 0.8 mg of X-gal (Sigma Chemical, St. Louis, MO, USA). The plates were incubated overnight at 37°C. Plates were checked for the presence of blue (pBluescript only) and white (pBluescript containing insert) colonies.

Minipreps

From the incubated plates described above, white colonies were picked and grown in 5 ml of liquid LB media supplemented with 100 μg/ml of ampicillin overnight. Miniprep reactions were performed to isolate plasmids and screen for proper insert size. One ml of bacterial culture was centrifuged at 15,000 x **g** for 30 seconds in a 1.5 ml test tube and the supernatant was poured off. The pelleted cells were resuspended in 100 μl of resuspension buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A), 200 μl of lysis buffer P2 (200 mM NaOH, 1% SDS) was added, and the solution was mixed and incubated at room temperature for five minutes. Finally, 150 μl of neutralization buffer P3 (3.0 M potassium acetate pH 5.5) was added, the solution was mixed and then incubated at room temperature for 15 minutes. The solutions were centrifuged at 15,000 x **g** for 5 minutes and 410 μl of the supernatant was transferred to a new tube. The plasmid DNA was precipitated by the addition of 500 μl of isopropanol. The resulting solution was incubated at room temperature for 15 minutes and then centrifuged at 15,000 x **g** for 10 minutes. The supernatant was removed and the DNA pellet was resuspended in 75 μl of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Plasmids were digested by adding 5 μl of miniprep DNA to a reaction mix containing *Bam* H I and *Xba* I as described above and incubated

at 37°C for one hour. The digestion products were loaded onto a 1% agarose gel containing ethidium bromide to visualize the fragment sizes as previously described. Miniprep cultures that resulted in digestion products of approximately 830 bp were used for plasmid isolation and for further characterization.

Plasmid isolation

Plasmids were isolated and purified from the transformed cells by midi-preps (Qiagen, Valencia, CA, USA). Erlenmeyer flasks (300 ml) containing 75 ml of LB media with 100 μg/ml ampicillin were inoculated with 100 μl of miniprep cultures from above. Cells were grown at 37°C overnight with shaking at 250 rpm. The cells were pelleted from the media by centrifuging at 5,000 x **g** for 5 minutes. The cells were resuspended in 5 ml of buffer P1, 5 ml of buffer P2 was added, the cells were mixed, and incubated at room temperature for five minutes. Five ml of buffer P3 was added, mixed, and incubated at 4°C for 15 minutes. The resulting solution was centrifuged at 12,000 x **g** for 30 minutes and the supernatant was added to the supplied QiaTip 100 column that had been equilibrated with 4 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH7.0, 15% isopropanol, 0.15% Triton X-100). The column was washed twice with 10 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol) and the plasmid eluted from the column with

5 ml of buffer QF (1.25 M NaCl, 50 mM Tris-Cl pH 8.5, 15% isopropanol). Plasmid DNA was precipitated by addition of 3.5 ml of isopropanol to the eluted plasmid solution and centrifugation at 20,000 x **g** for 30 minutes. The supernatant was removed and the DNA was resuspended in 50 µl of H_2O. Purified plasmids were sequenced to determine T3 promoter position and fidelity of the N^{pro} sequence. One plasmid, BS14, was determined to contain the correct insert and a sequence identical to that previously reported for BVDV isolate 890 (Ridpath and Bolin, 1995). This plasmid was used as a control plasmid for the subsequent generation of N^{pro} mutants.

Site-directed mutagenesis of N^{pro}

Site-directed mutations were introduced at amino acid positions 67, 69, 130, 168, and 169 using specific primers listed in Table 3. Each mutation created a specific restriction site to verify the substitution of an amino acid. In Figure 2, the position of each amino acid substitution with respect to the BVDV open reading frame is shown. The pBluescript control plasmid BS14 was used as template for the site-directed mutational PCR reactions. In order to generate inserts containing the desired mutation, the fragments for cloning were assembled in two separate reactions. The first reaction used primer 386 *Bam* H I as well as the mutation complement primer. The second reaction contained both the mutation primer and the complement primer 1216

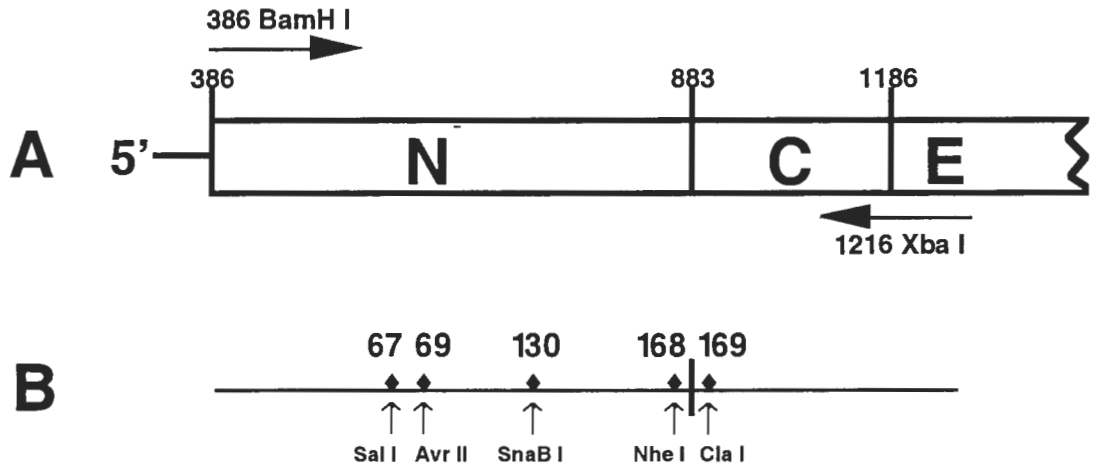

Figure 2. Illustration of the BVDV open reading frame encoded proteins and amino acid mutation positions. (A) Arrangement of BVDV genome, beginning with the 5' untranslated region and continuing through the N terminal protease (N^{pro}), capsid protein (C), and glycosylated envelope protein (E^{rns}). The nucleic acid positions are indicated for the respective protein start sites. PCR primers 386 BamH I and 1216 Xba I are shown to illustrate the region encoded in plasmid inserts. (B) Amino acid residues that are targeted for site-directed mutagenesis are indicated by ◆. The restriction site that is created within each mutation is noted by arrows.

Xba I. The two fragments were generated by PCR, as described above, were partially purified by Microcon-100 (Amicon, Inc., Beverly, MA, USA) centrifugation, and digested separately with the restriction enzyme that cut at the introduced restriction site. After phenol/chloroform extraction, the two

insert fragments were ligated overnight with T4 DNA ligase at 14°C as described above. The ligated insert and pBluescript II KS were then digested with *Bam* H I and *Xba* I and ligated overnight with T4 DNA ligase at 14°C. In Figure 3, the site-directed mutagenesis process is shown. The ligated plasmids were used to transform competent DH5α *E.* coli. Cells were grown on LB agar plates supplemented with ampicillin and X-gal as described above. White colonies were picked from the plates and grown in 5 ml of liquid LB media supplemented with ampicillin. These cultures were used for miniprep plasmid DNA isolation and screening for 830 bp inserts as described above. Cultures that were found to contain proper size inserts were grown in 75 ml of liquid LB media supplemented with ampicillin and the plasmids were isolated and purified by the midi-prep procedure described above. All plasmids were sequenced to determine T3 promoter position, fidelity of the open reading frame, and the presence of the desired mutation. Standard mutation symbols were used, with the first letter representing the original amino acid residue, the number indicating the amino acid position, and the last letter representing the amino acid that was substituted at this position.

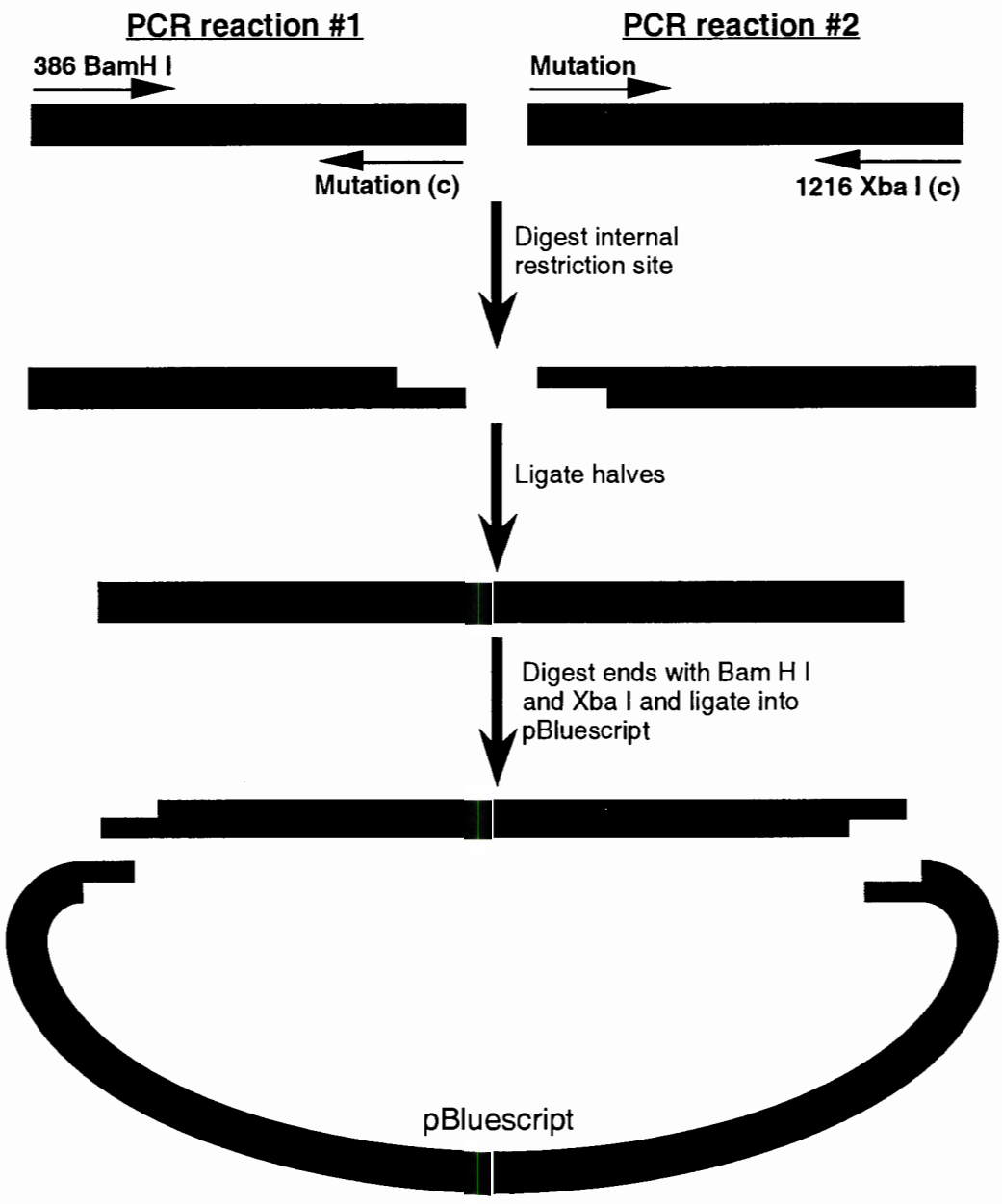

Figure 3. Creation of amino acid substitutions via site-directed mutagenesis. Insert segments were amplified by PCR, digested, and ligated. Complete inserts were ligated into pBluescript. See text for complete reaction reagents and conditions.

Error prone PCR

Additional mutations were introduced into N^{pro} by error prone PCR (Cadwell and Joyce, 1992; Ling and Robinson, 1997). The fidelity of the PCR reaction was reduced by doubling the *Taq* DNA polymerase (Boehringer Mannheim Corp., Indianapolis, IN, USA) concentration to 5 units, increasing the $MgCl_2$ concentration to 7 mM, and decreasing primer concentrations to 30 pM each. Primers 386 *Bam* H I and 1216 *Xba* I were used to amplify the insert containing region from the control plasmid BS14. PCR products were visualized on a 1% agarose gel with ethidium bromide to determine product size. PCR reactions were partially purified by Microcon-100 (Amicon, Inc., Beverly, MA, USA) centrifugation, digested with *Bam* H I and *Xba* I, cloned into pBluescript II KS, and used to transform DH5α *E. coli*. Transformed cells were plated on LB agar plates supplemented with ampicillin and X-gal and incubated overnight. White colonies were picked, grown, and plasmids screened by the miniprep procedure. Cultures that contained roughly 830 bp inserts were grown for plasmid isolation and purification by the midiprep procedure. Plasmids were sequenced to determine the location of the mutation, verify that only one amino acid substitution per insert was generated, and verify the open reading frame.

In vitro translation

For visualization of the autoproteolytic activity of N^{pro}, the above constructed control and mutant plasmids were translated in a cell-free *in vitro* transcription coupled translation reaction using wheat germ lysates (Promega, Madison, WI, USA). Transcription was directed from the T3 promoter and translation was performed in the presence of [^{35}S] methionine and [^{35}S] cysteine. The reactions were assembled by mixing 25 µl of wheat germ extract, 2 µl of reaction buffer, 1 µl of T3 RNA polymerase, 1 µl of amino acid mixture minus methionine, 2 µl of [^{35}S] methionine, 2 µg of plasmid DNA template and water to a final volume of 50 µl. The translation reactions were incubated at 30°C for 90 minutes and then treated with 0.2 mg/ml of RNase (Boehringer Mannheim Corp., Indianapolis, IN, USA) for 30 minutes at 30°C. Following addition of an equal volume of 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (100 mM Tris-HCl pH6.8, 200 mM DTT, 4% SDS, 0.2g bromophenol blue, 20% glycerol), the solutions were denatured by boiling. Translation products were visualized by electrophoresis on a 12.5% SDS-PAGE resolving gel [9.3 ml of 29:1 acrylamide: bis-acrylamide 40% solution (Amresco, Solon, OH, USA), 11.2 ml of 1 M Tris-HCl pH 8.7, 150 µl of 20% SDS, 7.8 ml of H_2O, 25 µl TEMED (Sigma Chemical, St. Louis, MO, USA) and 100 µl of 10% ammonium persulfate] with a 4% stacking gel [1 ml of 29:1 acrylamide: bis-

acrylamide 40% solution (Amresco, Solon, OH, USA), 1.25 ml of 1 M Tris-HCl pH 6.9, 50 µl of 20% SDS, 7.1 ml of H_2O,·25 µl of TEMED (Sigma Chemical, St. Louis, MO, USA) and 50 µl of 10% ammonium persulfate]. Following electrophoresis, the gel was fixed in a 60% H_2O: 30% ethanol: 10% acetic acid solution for one hour and dried at 65°C for four hours under vacuum. The gel was then exposed overnight at -80°C to X-Omat AR autoradiography film (Kodak, Rochester, NY, USA).

RESULTS

N^{pro} sequence comparison

DNA sequences of N^{pro} representing five isolates each of BVDV1, BVDV2, BDV, and CSFV were obtained. The N^{pro} DNA sequences were converted into amino acid sequences for sequence comparison. The amino acid sequences representing N^{pro} of 20 pestivirus isolates were aligned and conserved regions were identified. The results are displayed in Figure 4. The overall sequence identity among the 20 pestivirus isolates was 61.7%. The sequence identity among the five BVDV1 isolates was 85.9%, among the five BVDV2 isolates was 81.1%, among the five BDV isolates was 93.0%, and among the five CSFV isolates was 88.1%. There were many conserved regions throughout the N^{pro}, but significant regions (three or more amino acids) included amino acids: 1-3; 9-12; 40-49; 67-70; 78-82; 84-87; 95-99; 101-104; 119-123; 161-164. Conserved amino acid residues of N^{pro}, as revealed by this alignment, were selected as targets for site-directed mutagenesis.

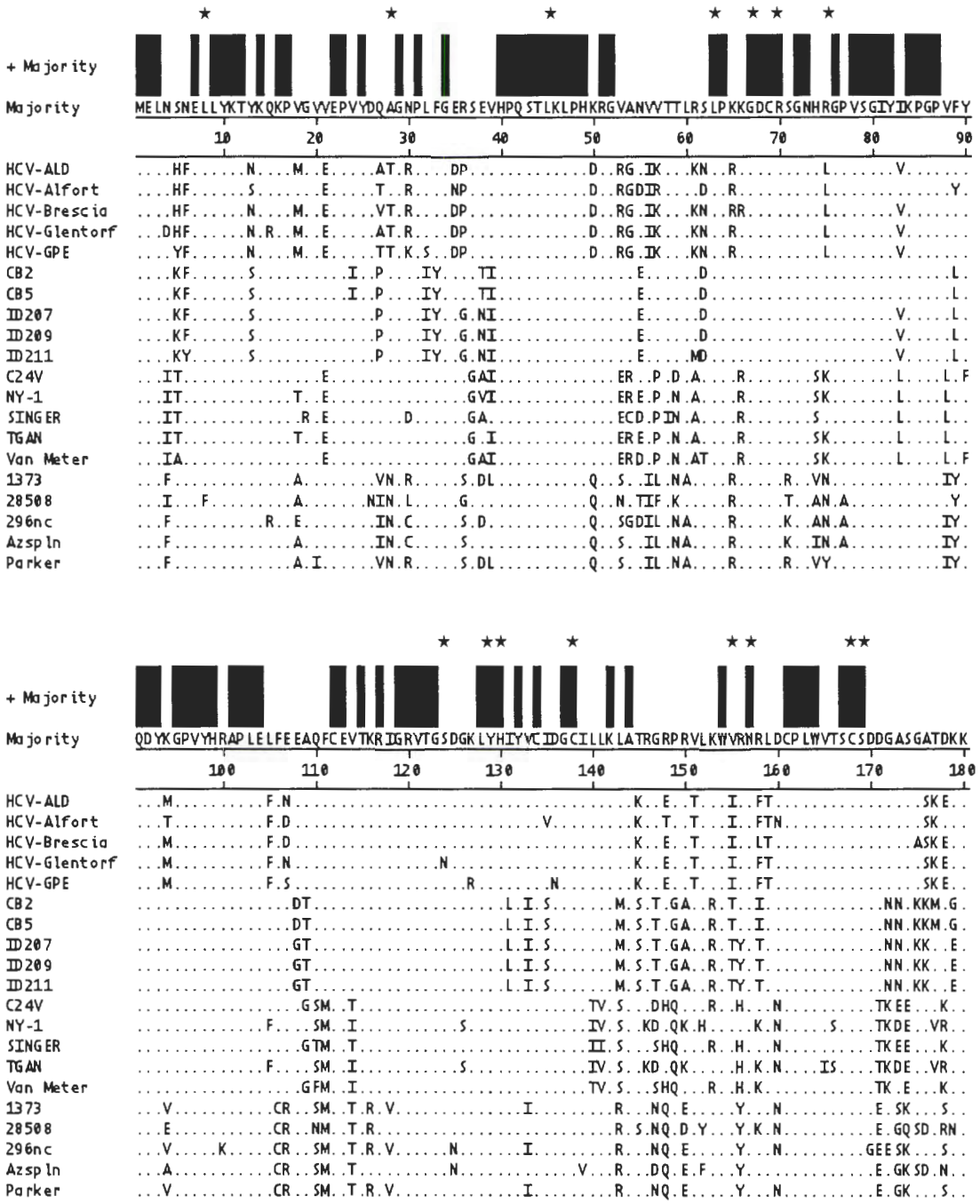

Figure 4. Comparison of amino acid sequences of pestivirus N^{pro}. Five isolates each of CSFV, BDV, BVDV1, and BVDV2 are compared. Conserved amino acids are indicated within each sequence by periods (.). Areas of conservation are indicated above the consensus sequence by black boxes. The amino acids that were mutated in this study are indicated by stars (★) above the conserved boxes.

Colony screening and mutant selection

Site-directed mutagenesis resulted in the creation of five plasmids containing single amino acid substitutions in N^{pro}. The resulting plasmids were designated according to the amino acid substitution that was created in N^{pro}: G67V, C69T, H130V, C168A, and S169I. In addition, a plasmid containing a 19 amino acid deletion within the N^{pro} from position 69 to 88 was isolated and designated 69DEL. Error prone PCR generated ten plasmids containing single amino acid substitutions in N^{pro} and were designated: L8F, N28S, K46R, L63P, N75Y, S124C, Y129C, C138R, V155L and N157S. In addition, three plasmids containing two amino acid substitutions per N^{pro} coding region were identified and designated as K16R - I135V, Q50R - M111V, and V121A - I133S.

In vitro translation

In vitro translation products of the control and mutant plasmid constructs were separated on 12% SDS-PAGE gels. The gels were dried and exposed overnight to autoradiograph film. The resulting gel images are displayed in Figures 5 and 6. The control plasmid BS14 translation products were 20 kDa (N^{pro}) and 14 kDa (capsid) in size, demonstrating that the

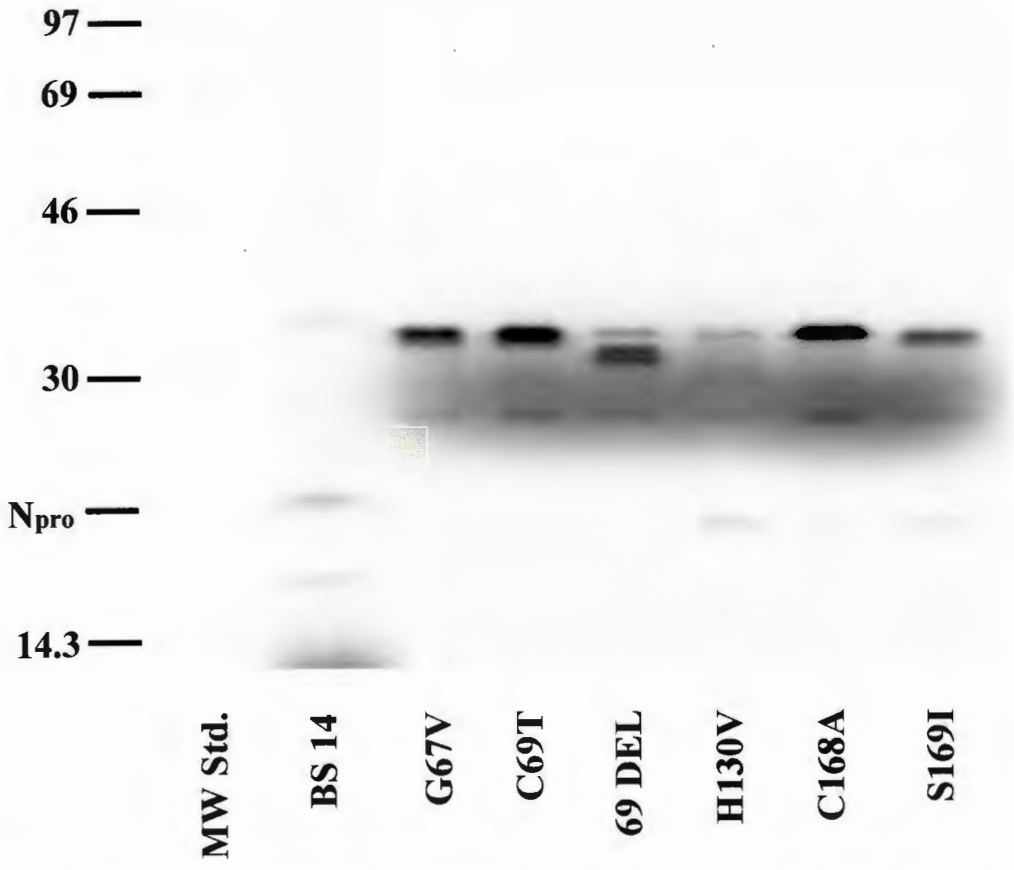

Figure 5. Autoradiograph of *in vitro* translation products from plasmid constructs generated from site-directed mutagenesis of N^{pro}. The amino acid substiutions that were created at selected residues are indicated. A 37 kDa precursor protein, containing N^{pro} capsid, and truncated E^{rns}, was observed in each translation. The self-cleavage of N^{pro} generated a 20kDa protein. Absence of a 20 kDa band indicates that the amino acid substitution prevented self-cleavage of N^{pro}.

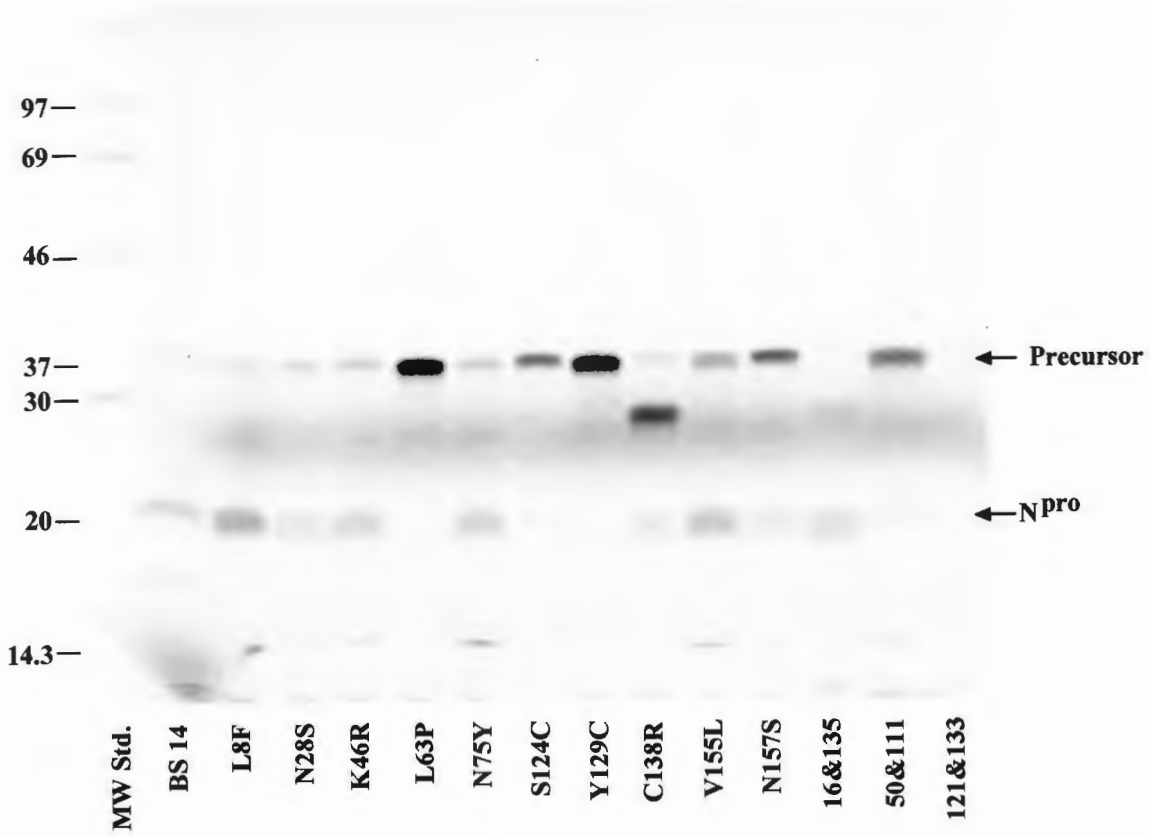

Figure 6. Autoradiograph of *in vitro* translation products of plasmid constructs generated by error prone PCR mutagenesis of N^{pro}. The amino acid substitutions that were created are indicated. A 37 kDa precursor protein containing N^{pro}, capsid, and truncated E^{rns}, was observed in each translation. The self-cleavage of N^{pro} generated a 20 kDa protein. Absence of a 20 kDa band indicates that the amino acid substitution prevented self-cleavage of N^{pro}.

unaltered N^{pro} possessed autocatalytic activity. A faint 37 kDa precursor protein product was also observed representing the uncleaved N^{pro}, C, and truncated E^{rns}. Plasmid H130V translation products were 20 kDa and 14 kDa in size, with a faint 37 kDa precursor protein band also observed. The cleavage patterns of the H130V translated proteins are similar to the cleavage patterns of the control plasmid BS14 translated proteins. Self-cleavage of N^{pro} (20 kDa) was not detected in translation products from plasmids G67V, C69T, and 69DEL, despite producing prominent 37 kDa precursor proteins. The shift in size of the precursor band from 69DEL can be attributed to the 19 amino acid deletion. Translated N^{pro} from plasmids C168A and S169I partially cleaved. Faint N^{pro} and C bands were observed, but a prominent precursor band was also produced by translation of both plasmids C168A and S169I.

Translation products from error prone PCR mutation plasmids L8F, N28S, K46R, N75Y V155L, and V121A - I133S produced cleavage patterns similar to plasmid BS14. The error prone PCR mutation clones L63P, S124C, and Y129C did not generate a 20 kDa protein (N^{pro}) but a 37 kDa precursor protein was produced in each translation reaction. Partial cleavage of N^{pro} and C was observed with translation products from plasmids C138R, N157S, K16R - I135V, and Q50R - M111V. Table 4 displays a summary of the N^{pro} cleavage patterns.

Table 4. N^{pro} amino acid substitutions were generated by site-directed mutagenesis and error prone PCR. The mutated N^{pro} encoding regions were cloned into pBluescript for *in vitro* translation. Cleavage of N^{pro} from the precursor protein was observed by SDS-PAGE gel separation.

Mutation	N^{pro} cleavage	Mutation	N^{pro} cleavage
L8F	+	H130V	+
N28S	+	C138R	+/-
K46R	+	V155L	+
L63P	—	N157S	+/-
G67V	—	C168A	+/-
C69T	—	S169I	+/-
69 DEL	—	K16R - I135V	+/-
N75Y	+	Q50R - M111V	+/-
S124C	—	V121A - I133S	+
Y129C	—		

+ indicates N^{pro} self-cleavage from precursor protein.

— indicates that N^{pro} self-cleavage was prevented.

+/- indicates that N^{pro} self-cleavage was partially prevented.

DISCUSSION

The work described in this study was performed to further characterize the catalytic unit of the amino terminal protease (N^{pro}) of BVDV. This hopefully will lead to the determination of the function of this protease. The catalytic unit is responsible for the self-cleavage of the protease. Catalytic units are composed of amino acid residues that fold into a conformationally active site and function in peptide bond cleavage. Catalytic dyads, triads, and tetrads have been reported for both cellular and viral-encoded proteases (Rawlings and Barrett, 1993). These catalytic units have been used to classify proteases into families of serine, cysteine and aspartic acid proteases (Rawlings and Barrett, 1993).

Amino acid comparisons of cellular and viral-encoded proteases with N^{pro} had previously identified some sequence homology between papain-related cysteine proteases and N^{pro} (Stark *et al.,* 1993; Rümenapf *et al.*, 1998). In these previous studies, several conserved amino acids were mutated to identify catalytic residues of N^{pro} but identification of the catalytic unit was not determined (Wiskerchen *et al.,* 1991; Rümenapf *et al.,* 1998).

In this study, the N^{pro} sequence of 20 pestivirus isolates was compared to identify conserved regions that could represent functional amino acids of N^{pro} (Figure 4). This comparison identified motifs suggestive of both cysteine

and serine proteases. Conserved cysteine (Cys) and histidine (His) residues were identified in our N^{pro} sequence comparison. The spatial arrangement of some of these Cys and His residues resembled a cysteine protease catalytic unit. Sequence comparison also revealed conserved His, aspartic acid (Asp), and serine (Ser) residues that suggested possible catalytic amino acids of a serine protease. In addition, a glycine - serine - aspartic acid - glycine (Gly123- Ser124- Asp125- Gly126) motif in N^{pro} was found to be similar to the Gly- Asp- Ser- Gly motif found in many serine proteases (Rawlings and Barrett, 1994).

Based on amino acid conservation of N^{pro}, site-directed mutations were created at residues Gly67, Cys69, His130, Cys168, and Ser169 of N^{pro}. Amino acid substitutions at Gly67 and Cys69 prevented self cleavage of N^{pro}. Gly67 was conserved among all pestivirus isolates compared in this study, indicating that it may be a component of the catalytic unit. Gly67 was not suspected as a possible catalytic residue in previous studies. The prevention of N^{pro} cleavage by a Cys69 substitution was recently reported (Rümenapf *et al.,* 1998) and a Cys69 substitution in our study also prevented cleavage of N^{pro}. Data presented in this study confirms that Cys69 is required for N^{pro} cleavage. An additional N^{pro} mutation created by site-directed mutagenesis resulted in the deletion of 19 amino acids after Cys69. This deletion prevented self-cleavage of N^{pro}. A deletion of amino acids may render the

protease non-functional because of folding failure, altered conformation, or loss of functional residues.

Amino acid His130 was conserved among all pestivirus isolates compared in this study. In addition, His130 was found in a region containing two other conserved amino acids. Translation data indicated that an amino acid substitution at His130 did not prevent self-cleavage of N^{pro}. A previous study also reported that substitution of His130 failed to prevent cleavage of N^{pro} (Rümenapf *et al.,* 1998). The data presented here confirms the lack of involvement of His130 in the catalytic unit of N^{pro}.

Site-directed mutations at amino acids Cys168 and Ser169 resulted in partial prevention of N^{pro} self-cleavage. The amino acid substitution at Cys168, the C-terminal end of N^{pro}, was expected to eliminate N^{pro} cleavage because of alteration of the cleavage site. Proteases have been reported to display specificity for their cleavage site and alteration of the C-terminal amino acid of many proteases has been reported to eliminate autoproteolytic cleavage (Babé and Craik, 1997; Wellink and van Kammen, 1988). The amino acid substitution of Ser169, the N-terminal residue of the capsid protein, also resulted in decreased N^{pro} cleavage. The results from translation of plasmids C168A and S169I suggest that self-cleavage is independent of the amino acids contained at the cleavage site. An additional

mechanism, such as distance of the C-terminal end of the protease from the active site, may be responsible for selection of the cleavage site.

A second approach to identify functional amino acids involved the use of error-prone PCR to generate additional amino acid substitutions. Error-prone PCR generated amino acid substitutions at residues Leu63, Ser124, and Tyr129 that prevented self-cleavage of N^{pro}. Leu63 and Tyr129 were found to be conserved among the 20 pestivirus isolates compared in this study. Assuming these amino acids are conserved among all pestiviruses, mutation of these residues would be more likely to eliminate autoproteolytic cleavage. Description of catalytic activity associated with leucine or tyrosine residues was not found in the available literature. Ser124 was found to be conserved among 19 of 20 pestiviruses compared in this study. The CSFV isolate Glentorf did not contain a conserved serine residue, but instead contained an asparagine residue at this position. It is possible that Ser124 is a catalytic amino acid residue of N^{pro}, suggestive of a serine protease. However, previous studies reported that substitution of Ser124 did not affect cleavage (Wiskerchen *et al.,* 1991).

Error prone PCR resulted in the creation of amino acid substitutions at residues Leu8, Asn28, Lys46, Asn75, Val121, Ile133, and Val155 that did not prevent N^{pro} cleavage. From the above listed mutations that did not prevent self-cleavage of N^{pro}, only Lys46 represents a conserved amino acid among

all pestivirus isolates compared in this study. Mutation of conserved residues was predicted to diminish or eliminate cleavage of N^{pro}, but the mutation at Lys46 did not support this hypothesis. Lys46 is located within a conserved motif of ten amino acids (Figure 4). This motif contains more sequential conserved amino acids than any other region found in the sequence comparison of N^{pro} and could represent a portion of the catalytic pocket of N^{pro}. Additional studies are needed to determine the importance of this conserved motif.

This study has demonstrated that substitutions amino acids Leu63, Gly67, Ser124, and Tyr129 prevent self-cleavage of N^{pro}. Data presented here supports a previous report that mutation of amino acid Cys69 also prevents N^{pro} self-cleavage (Rümenapf *et al.,* 1998). Previous studies also found that amino acids Glu22 and His49 (Rümenapf *et al.,* 1998) and His40 and Trp164 (Wiskerchen *et al.,* 1991) were necessary for N^{pro} self-cleavage. However, those studies were not able to identify the catalytic unit of N^{pro}.

The data presented in this study indicates that N^{pro} resembles the serine protease chymotrypsin. In addition, this study proposes that the His49/Asp68/Ser124 catalytic triad of N^{pro} is similar to the His/Asp/Ser catalytic triad of chymotrypsin. The majority of viral proteases that have been characterized are either cysteine or serine proteases. In this study, mutations introduced at both cysteine and serine residues prevented

cleavage of N^{pro}. In addition, mutations at other amino acids indicated other possible members of the catalytic unit. A previous study found that His49 was essential for N^{pro} cleavage (Rümenapf *et al.,* 1998). This study proposes that His49 is the first member of the catalytic triad. The second member of the proposed catalytic triad, aspartic acid, is present at amino acid position 68. Although this amino acid was not selected for mutation, mutations at both Gly67 and Cys69 eliminated cleavage of N^{pro}. These mutations flanked Asp68 and may have disrupted the catalytic function of Asp68. The third member of the proposed catalytic triad is Ser124. Results of this study demonstrated that mutation of Ser124 resulted in loss of N^{pro} cleavage. The amino acids of the His49/Asp68/Ser124 catalytic triad fold into a three-dimensional catalytic unit to cleave N^{pro} from C.

Additional support for the newly proposed protease characterization and catalytic unit identification is provided by a conserved motif around the active Ser124 residue. It has been reported that glycine residues are conserved near the catalytic serine residue of serine proteases. In addition, a conserved Gly-Asp-Ser-Gly motif is found in many serine proteases, although slight variations exist (Rawlings and Barrett, 1994). A similar conserved motif, Gly-Ser-Asp-Gly, was identified during the sequence analysis of N^{pro} in this study. Mutation of Tyr129, adjacent to this Gly-Ser-Asp-Gly conserved motif, eliminated self-cleavage of N^{pro}. This amino acid

substitution may have altered the structural conformation of the Ser124 motif that is essential for N^{pro} self-cleavage.

Future characterization of the N^{pro} autoprotease by additional mutagenic studies or 3-dimensional structural analysis will confirm whether N^{pro} is a chymotrypsin-like serine protease. The findings presented here provide a greater understanding of N^{pro} and will aid in future pestivirus and viral protease research.

GENERAL CONCLUSIONS

This study compared the N-terminal protease (N^{pro}) of BVDV type 1 and 2, CSFV, and BDV in order to identify conserved amino acid residues. This analysis revealed conserved regions that were compared to amino acid sequences of viral and cellular proteases to identify catalytic amino acids and potential catalytic units. Amino acid sequence analysis of N^{pro} revealed conserved cysteine (Cys) and histidine (His) residues, suggestive of a cysteine protease. Amino acid sequence analysis also revealed conserved His, aspartic acid (Asp), and serine (Ser) residues, suggestive of a chymotrypsin-like serine protease. A conserved glycine- serine- aspartic acid- glycine motif was discovered that also suggested similarity to the serine protease chymotrypsin.

Both site-directed mutagenesis and error prone PCR resulted in amino acid substitutions within N^{pro}. These mutated N^{pro} coding regions were translated *in vitro* and the effect of the amino acid substitution on N^{pro} self-cleavage was determined. This study demonstrated that mutations at amino acids Leu63, Gly67, Cys69, Ser124, and Tyr129 prevented self-cleavage of the N^{pro} autoprotease. This study proposes that amino acids His49, Asp68, and Ser124 represent the catalytic triad of N^{pro}. This catalytic triad resembles that of the serine protease chymotrypsin. Although a definitive protease

characterization cannot be made, the data presented in this study provides a better understanding of the autoproteolytic activity of N^{pro}.

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